Bacillus thuringiensis subsp. *israelensis* Cyt1Aa synergizes Cry11Aa toxin by functioning as a membrane-bound receptor

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***Bacillus thuringiensis* subsp. *israelensis* produces crystal proteins, Cry (4Aa, 4Ba, 10Aa, and 11Aa) and Cyt (1Aa and 2Ba) proteins, toxic to mosquito vectors of human diseases. Cyt1Aa overcomes insect resistance to Cry11Aa and Cry4 toxins and synergizes the toxicity of these toxins. However, the molecular mechanism of synergism remains unsolved. Here, we provide evidence that Cyt1Aa functions as a receptor of Cry11Aa. Sequential-binding analysis of Cyt1Aa and Cry11Aa revealed that Cyt1Aa binding to *Aedes aegypti* brush border membrane vesicles enhanced the binding of biotinylated-Cry11Aa. The Cyt1Aa- and Cry11Aa-binding epitopes were mapped by means of the yeast two-hybrid system, peptide arrays, and heterologous competition assays with synthetic peptides. Two exposed regions in Cyt1Aa, loop β6-αE and part of β7, bind Cry11Aa. On the other side, Cry11Aa binds Cyt1Aa proteins by means of domain II-loop α8 and β-4, which are also involved in midgut receptor interaction. Characterization of single-point mutations in Cry11Aa and Cyt1Aa revealed key Cry11Aa (S259 and E266) and Cyt1Aa (K198, E204 and K225) residues involved in the interaction of both proteins and in synergism. Additionally, a Cyt1Aa loop β6-αE mutant (K198A) with enhanced synergism to Cry11Aa was isolated. Data provided here strongly indicates that Cyt1Aa synergizes or suppresses resistance to Cry11Aa toxin by functioning as a membrane-bound receptor. *Bacillus thuringiensis* subsp. *israelensis* is a highly effective pathogenic bacterium because it produces a toxin and also its functional receptor, promoting toxin binding to the target membrane and causing toxicity.**

receptor interaction | binding epitopes | insect resistance | mode of action

Bacillus thuringiensis (Bt) is an attractive alternative to chemical insecticides because it is not toxic to vertebrates and to nontarget insects. One strain, *Bt* subsp. *israelensis* (Bti), has been used for >2 decades for mosquito control. Bti produces four crystal proteins (Cry) (4Aa, 4Ba, 10Aa, and 11Aa) and two Cyt (1Aa and 2Ba) proteins (1) that show toxicity to mosquito vectors of human diseases such as dengue, yellow fever, and malaria.

A major threat to the use of Bt is the appearance of insect resistance, which has been documented in lepidopteran insects (2); however, no resistance has been observed in the field in mosquito species controlled with Bti (3, 4). In contrast, resistant mosquito populations have been selected in the field with *Bacillus sphaericus*, another mosquitocidal bacterium that produces another toxin, Bin (5).

The lack of resistance to Bti is due to the presence of the Cyt1Aa protein in the crystal (6, 7). *Culex quinquefasciatus* populations resistant to Cry4A, Cry4B, or Cry11Aa have been selected under laboratory conditions, but mosquitoes resistant to Cry toxins could not be selected in the presence of Cyt1Aa toxin (8). Moreover, Cyt1Aa overcomes the resistance of the *C. quinquefasciatus* Cry-resistant populations (6). Recently, it was demonstrated that Cyt1Aa delays the evolution of resistance to Cry11Aa in *C. quinquefasciatus* (7). In addition, synergism between Cyt1Aa and the Cry proteins of Bti has been observed (9, 10); the activity of the Bti crystals is much higher than that of the isolated proteins (10, 11). Interestingly, Cyt1Aa also synergizes the mosquitocidal toxicity of the *B. sphaericus* Bin toxin, and confers sensitivity to this toxin to the naturally insensitive mosquito species *Aedes aegypti* (12, 13).

However, the molecular mechanism of synergism is unknown. This knowledge could provide strategies for coping with potential resistance problems. Cry and Cyt are pore-forming proteins; nevertheless, their three-dimensional structure is different. Cry toxins are composed of three functional domains: an α-helical domain involved in membrane insertion (domain I) and two β-sheet domains (domains II and III) involved in receptor interaction (14). In particular, the exposed loop regions in domain II are involved in receptor binding (14). Cyt proteins, on the other hand, have a single α–β domain composed of two outer layers of α-helix hairpins wrapped around a β-sheet (15). Both proteins are solubilized in the gut of susceptible dipteran insects and proteolytically activated by midgut proteases. For the Cry11Aa protoxin, proteolytic activation involves amino-terminal processing and intramolecular cleavage, leading to two fragments of 36 and 32 kDa that remain associated and retain insect toxicity (16).

Cry toxins bind to specific protein receptors in the microvilli of midgut epithelial cells, inducing toxin oligomerization and subsequent insertion into the membrane-forming lytic pores, causing cell swelling and lysis (14). In contrast, Cyt toxins do not bind to protein receptors and directly interact with membrane lipids inserting into the membrane and forming pores (17–19) or destroying the membrane by a detergent-like interaction (20). The toxicity of Cyt1Aa to mosquito larvae is, on average, one order of magnitude lower than that of Cry4 or Cry11Aa toxins (21).

Bacteria have developed various strategies for pathogenesis. In Bti and *Bacillus anthracis*, numerous toxins are produced, each with a different mode of action (1, 22). Alternatively, in the enteropathogenic *Escherichia coli*, these bacteria inject a translocated intimin receptor into host cells that then act in attachment of the bacteria to the host cells (23). Potentially, in the case of bacteria that rely on toxins for their pathogenesis, bacteria also could have developed another strategy: production of multiple proteins, one of which can then serve as a midgut receptor for the other toxins produced by these bacteria. In this article, we show that the Cyt1Aa protein functions as a receptor for the Cry11Aa toxin. We dem-

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Abbreviations: BBMV, brush border membrane vesicles; Bt, *Bacillus thuringiensis*; Bti, *Bacillus thuringiensis* subsp. *israelensis*; Cry, crystal proteins; HRP, horseradish peroxidase; LC_{50}, lethal concentration for 50 percent; PA-Seph, protein A-Sepharose CL-4B beads; SF, synergism factor.

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onstrate that Cry11Aa interacts with Cyt1Aa by the same domain II regions that are involved in receptor interaction. Furthermore, we show that Cry11Aa residues S259 and E266, located in loop α8 of domain II and Cyt1Aa residues K198, E204, and K225, are involved in the interaction between these proteins. Our data suggests that this interaction explains the synergism between the Cyt1A and Cry11A proteins.

Materials and Methods

Bacterial Strains. Bti HD567 was from the *Bacillus* Genetic Stock Center (Columbus, OH). Cry11Aa was produced in Bt CG6/pCG6 and Cyt1Aa in 4Q7/pWF45 (9, 24). Bt strains were grown in nutrient broth sporulation medium supplemented with 10 μg/ml erythromycin (25) shaken at 200 rpm and 30°C.

Purification of Cry11Aa and Cyt1Aa Proteins. Spores and inclusions produced by the Bt strains were harvested and washed three times with 0.3 M NaCl/0.01 M EDTA, pH 8.0. The pellet was suspended in 0.1% Triton X-100/300 mM NaCl/20 mM Tris·HCl, pH 7.2, and inclusions were purified by sucrose gradient centrifugation (26). Purified Cry11Aa inclusions were solubilized in 100 mM NaOH and activated with 1:50 N-p-Tosyl-L-phenylalanine chloromethyl ketone-treated bovine trypsin (Sigma-Aldrich) wt/wt for 2 h at 25°C obtaining two fragments of 36 and 32 kDa. Purified Cyt1Aa inclusions were solubilized in 50 mM Na_2CO_3/10 mM DTT, pH 10.5 and activated with 1:30 proteinase K (Sigma-Aldrich) wt/wt for 1 h at 30°C.

Preparation of Brush Border Membrane Vesicles (BBMV). BBMV from dissected midguts of fourth instar *A. aegypti* larvae were prepared as reported in ref. 27.

Sequential-Binding Analysis on Isolated BBMV. Ten micrograms of *A. aegypti* BBMV were preincubated 1 h at 25°C in 5 nM unlabeled and activated Cyt1Aa in 100 μl of binding buffer (PBS; 0.1% BSA wt/vol/0.1% Tween 20 vol/vol, pH 7.6), washed three times by centrifugation (10 min at 11,000 $\times$ *g*), and a membrane pellet was used afterward to analyze the binding of 5-nM biotinylated-activated Cry11Aa (1 h at 25°C) (RPN28, Amersham Pharmacia) as reported in ref. 28. Unbound biotinylated toxin was removed by centrifugation. Membrane pellet was loaded in SDS/PAGE and transferred to Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biosciences). The biotinylated toxin that remained bound to the vesicles was visualized by incubating with streptavidin-peroxidase conjugate (1:4,000 dilution) for 1 h and developed with SuperSignal chemiluminescence substrate (Pierce). Control membranes were BBMV without incubation with Cyt1Aa. Biotinylation of toxins did not affect their toxicity against *A. aegypti* larvae, as judged by bioassays performed with precipitated native or biotinylated Cry11Aa or Cyt1Aa as in ref. 11.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA plates, 96 wells, were incubated 12 h at 4°C with 5 μg/ml Cyt1Aa in 50 mM $NaHCO_3$, pH 9.6, followed by five washes with PBS/0.2% Tween 20. The plates were then incubated with PBS/0.5% gelatin (Bio-Rad)/0.2% Tween 20 for 1 h at 37°C and washed five times with buffer A (PBS/0.1% Tween 20). The ELISA plates were incubated with different concentrations of Cry11Aa or Cry1Ab (0.25–2 μg/ml) for 2 h at 37°C and washed again with buffer A. The Cry11Aa or Cry1Aa proteins bound to Cyt1Aa were detected with anti-Cry11Aa or anti-Cry1Ab antibody (1:3,000) for 2 h at 37°C, followed by a secondary goat-anti-rabbit-horseradish peroxidase (HRP) antibody for 1 h at 37°C. The HRP enzymatic activity was revealed with a freshly prepared substrate (40 mg of *o*-phenylenediamine and 18 ml of H_2O_2 in 100 ml of 100 mM NaH_2PO_4, pH 5.0). The enzymatic reaction was stopped with 6 M HCl, and the absorbance read at 490 nm with a Amersham Pharmacia LKB Ultraspec II. For competition assays, the biotinylated toxins were incubated in the presence of 10- or 100-fold molar excess of synthetic peptides. The sequence of the synthetic peptides used for competition assays are reported in the Table 2, which is published as supporting information on the PNAS web site, or in ref. 29.

Ligand Blot Assays. Two micrograms of Cry11Aa or Cyt1Aa proteins were separated in 10% SDS/PAGE and electrotransferred to Hybond-ECL membranes. After blocking, the membranes were incubated for 2 h in 5 nM of biotinylated Cyt1Aa or Cry11Aa, and the bound protein was revealed with streptavidin-peroxidase conjugate as above. For competition assays, the biotinylated toxins were incubated in the presence of 25- or 50-fold molar excess of synthetic peptides.

Immunoprecipitation Assays. Immunoprecipitation of Cry11A protein was performed as described in ref. 30. Anti-Cry11Aa antibody was coupled to protein A-Sepharose CL-4B beads (PA-Seph, Amersham Pharmacia). Cry11Aa toxin (10 μg) was incubated for 1 h at 25°C in PBS in the presence or absence of Cyt1Aa (10 μg). Cyt1Aa in PBS was included as a negative control. Then, 100 μl of anti-Cry11Aa-PA-Seph were added and incubated 12 h at 4°C. Alternatively, BBMV (50 μg of protein) were incubated for 1 h with 10 μg of Cyt1Aa at 25°C. Membranes were centrifuged 10 min at 11,000 $\times$ *g*, washed, and suspended in PBS. Cry11Aa (10 μg) was incubated with treated BBMV for 1 h at 25°C. BBMV were washed as above, and membrane pellets were solubilized for 1 h at 25°C in 100 μl of PBS/1 mM PMSF/40 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate/10 mM $CaCl_2$. The samples were centrifuged 10 min at 11,000 $\times$ *g*, and the supernatant containing the solubilized BBMV proteins were incubated for 12 h at 4°C with 100 μl of anti-Cry11Aa-PA-Seph. Negative controls were Cry11Aa-toxin incubated with BBMV not preincubated with Cyt1A, and BBMV preincubated only with Cyt1A without the addition of Cry11A. PA-Seph from all treatments were then pelleted and washed six times with PBS. The binding complex was dissociated from the beads by boiling in SDS-solubilization Laemmli buffer and separated in 12% SDS/PAGE. Proteins were transferred to Hybond-ECL membranes, blocked with skim milk (5%), and visualized by Western blot. The membranes were incubated with anti-Cry11Aa or anti-Cyt1Aa polyclonal antibodies (1:10,000) and then with a secondary goat anti-rabbit-HRP antibody (Sigma) (1:5,000). Blots were visualized by using the Super-Signal substrate (30). Other negative controls were Cry11Aa or Cyt1Aa toxins directly loaded in the gel and detected with anti-Cyt1Aa or anti-Cry11Aa polyclonal antibodies, respectively.

Plasmid Constructions. Synthetic full-length *cry11Aa* and *cyt1Aa* genes (GenBank accession nos. M31737 and X03182) and the different gene fragments were obtained by PCR with specific oligonucleotides designed with OLIGO4 (Molecular Biology Insights, Cascade, CO) program (Tables 3 and 4, which are published as supporting information on the PNAS web site). The PCR products of *cry11Aa* gene amplification were digested with SacI and PstI restriction enzymes (New England Biolabs) and cloned into plasmid pHybLex/Zeo (Invitrogen). The PCR products of *cyt1Aa* gene were digested with KpnI and SphI and cloned into plasmid pYESTrp2 (Invitrogen).

Yeast Two-Hybrid Assay. Yeast transformation was performed by using lithium acetate (31). The L40 yeast strain [MAT*a* *his*3Δ200 *trp1-90l leu2-3112 ade2* LYS2::(*4lexAop-HIS3*)URA3::(*8lexAop-lacZ*)GAL4] was cotransformed with 0.5 μg of each plasmid. Transformants were plated on media lacking tryptophan plus 300 μg/ml zeocin to secure the presence of both plasmids, eliminating the possibility of false negatives. Growth on media lacking tryptophan and histidine plus zeocin and 1 mM aminotriazole was used to detect potential interactions. Cells surviving on

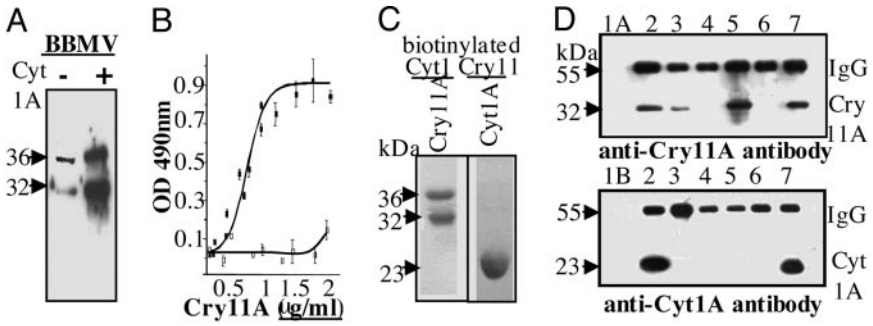

Fig. 1. Analysis of the interaction of Cry11Aa and Cyt1Aa proteins. (*A*) Sequential-binding analysis of Cyt1Aa and Cry11Aa to *A. aegypti* BBMV. BBMV were preincubated with 5 nM unlabeled Cyt1Aa, washed by centrifugation, and used afterward to perform the binding of 5-nM biotinylated Cry11Aa. (*B*) Binding analysis of Cry11Aa or Cry1Ab to Cyt1Aa by ELISA. ELISA plates were coated with Cyt1Aa and then incubated with different concentrations of Cry11Aa or Cry1Ab (0.25–2 μg/ml). Bound Cry11Aa (■) was detected with anti-Cry11Aa antibody and Cry1Ab (□) with anti-Cry1Ab antibody followed by a secondary goat-anti-rabbit-HRP antibody. (*C*) Ligand blot assays of biotinylated Cry11Aa or Cyt1Aa proteins (5 nM) to Cyt1Aa or Cry11Aa (2 μg), respectively, that were blotted into Hybond membranes. (*D*) Immunoprecipitation of Cry11Aa by using PA-Seph linked to anti-Cry11Aa antibody. The associated proteins were revealed with specific antibodies against Cry11Aa (*Upper*) or Cyt1Aa (*Lower*) in Western blot assays. Lane 1A, control of Cyt1A toxin directly loaded in the gel; Lane 1B, control of Cry11A directly loaded in the gel. The rest of the lanes were immunoprecipitated with anti-Cry11A-antibody, Lanes: 2, Cry11A plus Cyt1A in solution; 3, Cry11A in solution; 4, Cyt1A in solution; 5, Cry11A associated to BBMV; 6, Cyt1A associated to BBMV; 7, Cry11A bound to BBMV pretreated with Cyt1Aa. Cry11A antibody only reacts with the 32-kDa band of Cry11Aa toxin.

these plates were further screened for β-galactosidase activity by filter assay (32).

Cry11Aa Binding to Cellulose Membrane-Bound Peptides. Cellulose-bound peptides of Cyt1Aa were prepared by (Jerini Peptide Technologies, Berlin) by automated spot synthesis (33). The membrane-bound peptides were washed with ethanol and then with Tris-buffered saline (TBS; 50 mM Tris/137 mM NaCl/2.7 mM KCl, pH 8) before blocking for 16 h at 4°C with TBS/0.1% Tween 20/5% skim milk. The membrane was washed with TBS/0.1% Tween 20 before incubation with 20 μg/ml biotinylated Cry11Aa toxin in TBS/0.1% Tween 20 at 4°C for 4 h, detected with streptavidin-HRP conjugate (1:5,000) at room temperature, and finally visualized with SuperSignal chemiluminescence substrate.

Site-Directed Mutagenesis of Cyt1Aa Toxin. Mutagenesis of pWF45 plasmid was performed by using QuikChange XL kit (Stratagene). Appropriate oligonucleotides were synthesized for each mutant construction. Mutants were sequenced and transformed into acrystalliferous Bt strain 407.

Insect Bioassay. Mosquitocidal bioassays were performed against 20 early fourth-instar larvae in 100 ml of dechlorinated water. Different protein ratios of Cyt1Aa:Cry11Aa mixtures (1:1, 0.5:1, and 0.2:1) were assayed. Positive (Bti) and negative controls (dechlorinated water) were included in the bioassay, and larvae examined 24 h after treatment. The mean lethal concentration for 50 percent (LC_{50}) was estimated by PROBIT analysis with statistical parameters (34) after four independent assays (Polo-PC LeOra Software, Berkeley, CA). The theoretical toxicity of each ratio mixture was evaluated according to Tabashnik's equation (35), assuming a simple additive effect. The theoretical LC_{50} value is the harmonic mean of the intrinsic LC_{50} values of each component weighted by the ratio used in the mixture as follows:

$$LC_{50}\ (\text{Cyt1A} + \text{Cry11A}) = \left[\frac{r\text{Cyt1A}}{LC_{50}\ (\text{Cyt1A})} + \frac{r\text{Cry11A}}{LC_{50}\ (\text{Cry11A})}\right]^{-1}, \quad [1]$$

where *r*Cyt1A and *r*Cry11A are the Cyt1A and Cry11A protein proportions used in the final ratio of the mixture. LC_{50}(Cyt1A) and LC_{50}(Cry11A) are the LC_{50} values for each individual toxin. The synergism factor (SF) was calculated by dividing the theoretical toxicity by the observed toxicity of the mixture in bioassays. SF values >1 indicate synergism.

Determination of Binding Affinities by Competition ELISA. To determine apparent dissociation constants (K_d), Cyt1Aa or Cyt1Aa mutants K198A, E204A, or K225A (10 nM) were incubated with increasing concentrations of Cry11Aa toxin or Cry11Aa mutants S259A or E266A (from 0.1 nM to 1 μM) in 100-μl volume for 1 h at room temperature. The incubation mixtures were transferred to a 96-well ELISA plate previously coated with 2.5 μg of Cyt1Aa and processed as above. The concentration of Cry11Aa- or Cry11Aa-mutant toxins at which the half-maximal ELISA signal is detected corresponds to the apparent K_d (36).

Results

Binding and Interaction of Cry11Aa and Cyt1Aa Proteins. Previous work demonstrated that the Cyt1Aa protein synergizes the toxicity of the other Cry toxins in Bti (9). Further, together the Cyt1A and Cry11Aa proteins bind to the apical membrane in the same midgut region of the mosquito larvae (37). To analyze whether the Cyt1Aa enhances Cry11Aa binding, we performed a sequential-binding experiment with the Cyt1Aa and Cry11Aa proteins. Fig. 1*A* shows that binding of Cry11Aa to *A. aegypti* membranes was greatly enhanced by preincubation of BBMV with unlabeled Cyt1Aa. The binding of biotinylated Cry11A in the presence of Cyt1A was still saturable. However, higher concentrations of cold Cry11A are required to compete the binding (Fig. 6, which is published as supporting information on the PNAS web site), suggesting that Cyt1Aa-BBMV have more binding sites for Cry11Aa than untreated BBMV.

To determine whether this enhanced BBMV binding is facilitated by interaction of Cry11Aa and Cyt1Aa, the binding of these proteins was analyzed by different methodologies. Analysis of binding by ELISA showed a saturable binding of Cry11Aa to Cyt1Aa (Fig. 1*B*), in contrast to Cry1Ab, which showed no binding. In ligand blot assays, the biotinylated Cry11Aa binds to blotted Cyt1Aa, and the biotinylated Cyt1Aa binds to the blotted Cry11Aa (Fig. 1*C*).

Finally, coimmunoprecipitation studies demonstrated that Cry11Aa could interact with Cyt1Aa in solution and in the membrane-bound state. Cry11Aa was incubated with Cyt1Aa in solution or with Cyt1Aa previously bound to BBMV. Membranes were solubilized with 3-[(3-cholamidopropyl)dimethylammonio]-1-

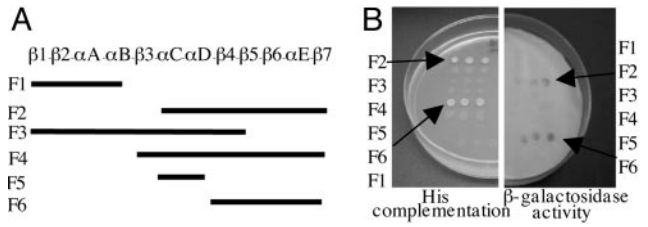

Fig. 2. Interaction between Cry11Aa and Cyt1Aa by yeast two-hybrid system. (*A*) Description of Cyt1Aa constructions cloned in pYESTrp2. (*B*) Interaction by cotransformation of L40 yeast strain with pHbyLEX/Zeo containing domains II and III of Cry11Aa and pYESTrp2 containing the different Cyt1Aa fragments. Positive clones were assayed by histidine auxotrophy complementation and for β-galactosidase activity in three independent colonies.

propanesulfonate detergent, and the Cry11Aa toxin was immunoprecipitated with an anti-Cry11Aa polyclonal antibody. The immunoprecipitates were separated by SDS/PAGE and analyzed for the presence of Cyt1Aa and Cry11A proteins by Western blot. Fig. 1*D* shows that Cry11A and Cyt1Aa are present in an interacting complex either in solution (lanes 2) or when Cyt1Aa was previously incorporated into BBMV (lanes 7). Negative control showed that Cyt1Aa was precipitated only in the presence of Cry11Aa (lanes 4 and 6). In addition, immunoprecipitation analysis of Cry3Aa and Cry1Ab toxins, using anti-Cry3Aa- or anti-Cry1Ab-PA-Seph, respectively, confirmed the specificity of Cry11A–Cyt1Aa interaction because Cyt1Aa was not coprecipitated with other Cry toxins (Fig. 7, which is published as supporting information on the PNAS web site).

Identification of Binding Epitopes. We hypothesized that Cyt1Aa and Cry11Aa are interacting through specific epitopes. As a first approach to identify the regions involved in their interaction, we used the yeast two-hybrid system (31). The full-length *cry11Aa* gene or the receptor-binding domains II and III were fused to the LexA DNA binding domain, whereas the full-length *cyt1Aa* coding region was fused to the B42 activation domain. Both Cry11Aa constructs bound the Cyt1Aa as demonstrated by complementation of his auxotrophic marker and production of β-galactosidase activity (data not shown). To narrow the region of the Cyt1Aa protein involved in binding to Cry11Aa domain II and III, six partially overlapping protein fragments of Cyt1Aa were fused to the LexA activator domain and tested for interaction with the domain II and III fragment of Cry11Aa (Fig. 2*A*). Only fragments F2 and F6 interacted with domains II and III of Cry11Aa (Fig. 2*B*). These two fragments share 105 residues in the C-terminal region from β4 to β7 (Fig. 2*A*).

To narrow further the binding region in Cyt1Aa, a library of overlapping peptides derived from the helix αC through the β7 sequence of Cyt1Aa was created by using the spot synthesis technique (33). Thirty-one peptides of 15 amino acids each (12 residues overlap with the previous peptide) were bound to nitrocellulose membrane and screened with biotinylated-Cry11Aa. Fig. 3*A* shows that the darkest signal was obtained with sequences $_{196}$EIKVSAVKE$_{204}$ (spots 16–20) and $_{220}$NIQSLKFAQ$_{228}$ (spots 23–26). These two regions were localized on a space-filled model of Cyt1Aa, which was obtained by comparison to the Cyt2A structure (15) (Fig. 3*C*). The residues $_{196}$EIKVSAVKE$_{204}$ are located in the highly exposed loop β6-αE, whereas $_{220}$NIQSLKFAQ$_{228}$ are located within β7. Both Cry11Aa-binding regions are in close proximity in the Cyt1A structure (Fig. 3*C*).

To determine the role of these residues in the interaction with Cry11Aa, synthetic peptides corresponding to these sequences were used in competition experiments by ELISA (Fig. 4*A*) and ligand blot assays (Fig. 4*B*). Both peptides completed the binding of Cyt1Aa and Cry11Aa proteins. A scramble peptide with the same amino acid composition as EIKVSAVKE but with a different sequence (IVKAVKEEV) used at the highest concentration (100 M excess) did not complete the binding of these proteins (Fig. 4*A*).

Finally, to identify the epitopes in Cry11Aa involved in interaction with Cyt1Aa, we performed competition-binding assays in the presence of synthetic peptides that correspond to the exposed loops of domain II of Cry11Aa (29) and two other amino acid sequences that are exposed (named β-4 and β-6) (29). Some of the exposed loop regions of domain II of Cry toxins are involved in toxin–receptor interaction (14, 29). Synthetic peptides corresponding to loop α8, loop2, and β4 of Cry11A competed with the binding of Cry11Aa to Cyt1Aa in ELISA assays (Fig. 4*C*) or in ligand blot assays (Fig. 4*D*), in contrast to loop 1, loop 3, and β6 peptides that did not inhibit this interaction, suggesting that domain II loop α8, loop2, and β4 regions of Cry11Aa toxin might be involved in the interaction of these proteins.

Identification of Residues Involved in Synergism and in Cry11Aa–Cyt1Aa Interaction. To further characterize the binding regions in both toxins, we analyzed the synergism and binding interaction of different Cyt1Aa and Cry11Aa mutants. Three Cry11Aa mutants (P261A, V262A, and E266A) in the loop α8 region, which also affected receptor-binding interaction, showed 3-, 5- and 20-fold lower toxicity to *A. aegypti*, respectively (29), whereas mutants S259A and N263A did not affect binding or toxicity (29). Preliminary bioassays in the presence of Cyt1Aa demonstrated that only Cry11Aa mutants E266A and S259A eliminated synergism (Fig. 8, which is published as supporting information on the PNAS web site). In addition, we isolated Cyt1Aa mutants in loop β6-αE and β7. All Cyt1Aa mutant proteins were stable to proteolysis and showed insecticidal against *A. aegypti* larvae (Fig. 9 and Table 5, which are published as supporting information on the PNAS web site).

The SF of different ratio mixtures of wild-type Cyt1Aa and Cry11Aa toxins was determined by bioassays against *A. aegypti* larvae. For the two wild-type proteins, the highest SF value of ≈18 was found at the 0.2:1 Cyt1Aa:Cry11Aa ratio (Table 1), in contrast

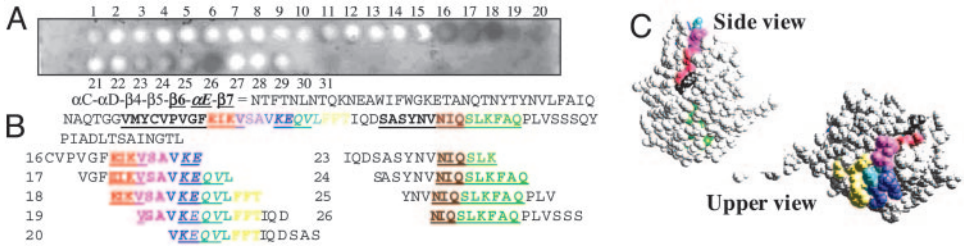

Fig. 3. Identification of the minimal region of Cyt1Aa involved in Cry11Aa interaction. (*A*) Immobilized overlapping peptides in membrane (synthesis SPOT) corresponding to the region of αC-β7 of Cyt1Aa were analyzed for binding of biotinylated Cry11A toxin. (*B*) Description of the overlapping peptides that bound Cry11Aa. β6 and β7 are underlined and bold, whereas helix-αE is underlined and italics. (*C*) Localization of the two identified regions (EIKVSAVKEQVLFFT and NIQSLKFAQ) in the Cyt2A structure. NIQ residues are drawn in black for clarity.

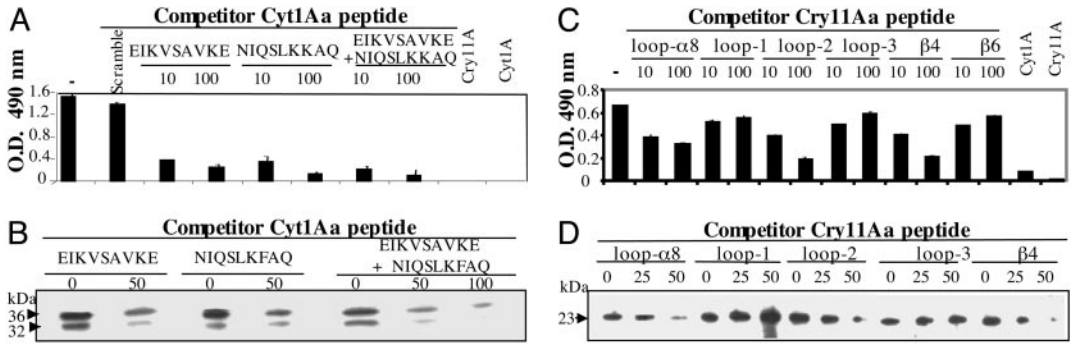

Fig. 4. Competition assays in the Cyt1Aa–Cry11Aa interaction with synthetic peptides. (*A*) Analysis of binding by ELISA in the presence or in the absence of 10–100 M excess of synthetic peptides EIKVSAVKE or NIQSLKFAQ. Negative control was a scramble peptide used at 100 M excess. ELISA plates were coated with 5 μg/ml Cyt1Aa, blocked and incubated with 0.8 μg/ml Cry11Aa. (*B*) Binding analysis by ligand blot assay. Binding of 5 nM biotinylated Cyt1Aa to 2 μg of Cry11Aa blotted into Hybond membranes was analyzed in the absence or presence of 50–100 M excess of Cyt1Aa synthetic peptides EIKVSAVKE or NIQSLKFAQ. Bound-labeled Cyt1Aa was visualized by streptavidin-peroxidase conjugate. (*C*) Analysis of binding of Cry11Aa toxin to Cyt1Aa by ELISA in the presence or absence of 10–100 M excess of synthetic peptides corresponding to the loops and exposed regions of Cry11Aa-domain II. (*D*) Ligand-blot assays of binding of 5 nM biotinylated Cry11Aa toxin to 2 μg of Cyt1Aa protein blotted into Hybond membranes. Synthetic peptides were used at 25 M or 50 M excess. In *A* and *C*, Cyt and Cry lanes are negative controls without the other protein. Numbers at top of ELISA histogram bars (*A* and *C*) and at top of ligand-blot bands (*B* and *D*) correspond to the molar excess of synthetic peptides used as competitors.

to SF values of 8 for 0.5:1 ratio or 3.5 for 1:1 ratio. At 0.2:1 mixture ratio, the Cry11Aa mutants S259A and E266A showed a SF of 2.5 and 2.3, respectively, whereas Cyt1Aa mutants E204A and K225A also showed a reduction in SF value, indicating that these mutations affected the synergistic activity. Furthermore, the mixture of mutants Cyt1Aa-K225A and Cry11Aa-S259A resulted in a very low toxicity without synergism. (Table 1 and Fig. 5). Interestingly, Cyt1Aa loop β6-αE mutant K198A showed an increase in SF, two times higher than that observed with wild-type Cyt1Aa (Table 1 and Fig. 5).

The binding of wild-type and mutant proteins was analyzed by ELISA. Fig. 5 shows that binding of these proteins correlates with the SF value of the different Cyt1Aa-Cry11Aa mixtures. Cyt1Aa-K198A mutant showed slightly higher binding to Cry11Aa, whereas Cyt1Aa mutants E204A and K225A showed reduced binding to Cry11Aa. Regarding to Cry11Aa mutants, S259A and E266A bound Cyt1Aa less efficiently than wild-type Cry11Aa. Finally, the binding of the two mutated proteins (Cyt1A-K225A and Cry11A-S259A) was severely affected (Fig. 5). Competition ELISA, which analyzed interaction of proteins in solution, was used to determine the apparent dissociation constants (K_d) (see *Materials and Methods*). Table 1 shows that Cry11Aa mutant S259A bound Cyt1Aa with a higher K_d, indicating that the binding affinity is 10 times lower than that of Cry11Aa, whereas E266A mutant showed a 3-fold reduced binding affinity to Cyt1Aa. Also, Cyt1Aa mutants E204A and K225A showed a 3- and 10-fold higher K_d, respectively, than Cyt1Aa. In the case of Cyt1Aa K198A, a slight decrease in the apparent K_d value was observed. The interaction of Cyt1Aa mutant K225A to Cry11Aa mutant S259A showed a K_d value 75-fold higher than that of wild-type proteins (Table 1). These data show that the decrease in binding of Cry11Aa or Cyt1Aa mutants correlated with the loss of synergism observed in bioassays.

Table 1. Toxicity of Cyt1Aa and Cry11Aa proteins, synergism factor, and binding affinities of mixtures of proteins at 0.2:1 ratio

Toxins Cyt1Aa:Cry11Aa	Predicted LC_{50}, ng/ml	Experimental LC_{50}, ng/ml	SF*	Apparent K_d,† nM
Wt:wt	227.3	12.3 (0.4–30.8)‡	18.5	0.4
Wt:E266A	650.3	286.0 (164.9–521.7)	2.3	1.2
Wt:S259A	179.0	70.9 (35.6–119.4)	2.5	4.0
K198A:wt	227.8	5.7 (0.3–14.2)	40.0	0.3
E204A:wt	202.6	25.8 (11.4–42.0)	7.9	1.5
K225A:wt	222.5	66.4 (42.3–111.4)	3.4	4.0
K225A:S259A	176.5	445.5 (375.9–515.9)	0.4	30.0
Cyt1Aa§	—	1244.6 (909.9–1423.1)	—	—
Cry11Aa§	—	235.9 (102.4–499.6)	—	—

*Synergism factor (Predicted LC_{50}/Experimental LC_{50}).
†Apparent dissociation constant obtained from ELISA competition assays.
‡95% fiducial limits.
§Toxicity of wild-type toxins tested alone.

Discussion

Bti is highly effective in mosquito control, but even though it has been extensively used for >20 years, insect resistance in field populations has not been observed (3, 4). The high efficacy of Bti is because of the production of multiple toxins with different modes of action. Further, the Cyt proteins in Bti synergize the toxic effect of Cry11A and Cry4 toxins and, even more, suppresses the resistance to these Cry toxins (6, 7). The results presented in this work are consistent with a model in which Cyt1Aa protein synergizes Cry11Aa toxicity by functioning as a receptor molecule.

We first showed that binding of Cry11Aa to *A. aegypti* BBMV was enhanced by membrane-bound Cyt1Aa (Fig. 1*A*), suggesting that Cyt1Aa is acting as a receptor for Cry11Aa. Then we demonstrated the specific and high-affinity interaction between Cyt1Aa and Cry11Aa in solution and membrane-bound conformation by different procedures (Fig. 1 *B–D* and Table 1). In

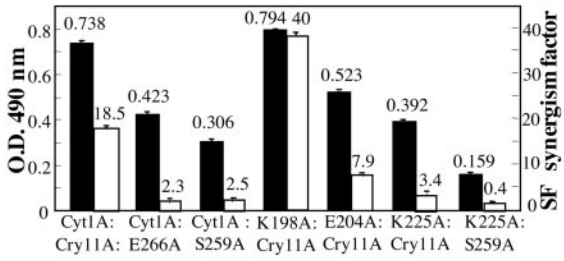

Fig. 5. Analysis of SF and binding interaction of different Cyt1Aa and Cry11Aa mutants. The binding of 0.4 μg/ml Cry11Aa to 5 μg of Cyt1Aa protein was analyzed by ELISA. Maximal absorbance at 490 nm is represented (filled bars). The synergism factor of different ratio mixtures of Cyt1Aa:Cry11Aa proteins was determined by calculating theoretical LC_{50} by Tabashnik's equation and experimental LC_{50} by bioassays against *A. aegypti* larvae (open bars).

contrast, Cry3A or Cry1Ab toxins do not interact with Cyt1A, correlating with the absence of synergism between these toxins and Cyt1A (Figs. 1*B* and 7).

The epitopes in Cyt1Aa that bind Cry11A were identified (Fig. 3). The role of loop β6-αE and part of β7 of Cyt1Aa in binding Cry11Aa was confirmed by heterologous competition assays with synthetic peptides corresponding to these regions (Fig. 4) and by site-directed mutagenesis (Fig. 5 and Table 1). Previously, it was suggested that Cyt proteins insert into the membrane by means of the β-sheets structures, leaving the β6-αE loop exposed (15).

Similarly, specific Cry11Aa epitopes involved in binding Cyt1Aa were identified (loop α8, β4, and loop 2) (Fig. 4 *C* and *D*). Surprisingly, the interaction of Cry11Aa with Cyt1Aa involves two epitopes that are also involved in Cry11Aa–mosquito receptor interaction because we showed previously that Cry11Aa interacts with its *A. aegypti* midgut receptor through domain II loop α8 and by β4 and loop 3 (29). These data may indicate that Cry11Aa binding to membrane-bound Cyt1Aa could result in the insertion of Cry11Aa into the membrane, just like the interaction of Cry11Aa with its natural receptor.

The binding of the Cry11Aa toxin to Cyt1Aa could constitute the basis for synergism. The mutants in the identified epitopes that affected binding affinity in the Cyt1Aa/Cry11Aa interaction were also affected in their synergism (Table 1 and Fig. 5). Although changes in the binding affinities (apparent K_d) were not large, there was a correlation between the reduction of binding and the reduction of synergism. In addition, we identified a Cyt1Aa mutant (K198A) that showed a reproducible slightly higher affinity to Cry11Aa (Table 1 and Fig. 5) and a correlative 2-fold higher synergism factor. In the case of the lepidopteran Cry1Aa toxin, a loop 2 mutation N372A also enhanced receptor affinity and correlated with higher toxicity toward the gypsy moth (38). This data suggest that the Cyt1Aa-K198A mutant could be very useful for producing Bti formulations with improved activity against mosquito larvae. It is anticipated that the loop regions of the Cry4A and 4B toxins, whose toxicity is also synergized by Cyt1Aa, could be involved in the interaction with Cyt1Aa (C.P., M.S., and A.B., unpublished data).

The combination of two mutants (Cyt1A-K225A and Cry11Aa-S259A) showed a 75-fold higher apparent K_d and no synergism of these toxins was observed; in fact, antagonism between these toxins was apparent (SF 0.4; Table 1). Neither mutant K225A nor S259A was affected in protease activation, binding to BBMV, or toxicity, indicating that protein structure was not affected (ref. 29; Fig. 9). These data suggest that interaction of both toxins involves more than one contact site and mutagenesis of at least two sites was necessary to observe a significant decrease in binding and synergism.

Cry11Aa-E266A located in loop α8 is also affected in binding to its midgut receptor in *A. aegypti* and had lower toxicity, whereas mutant S259A had no effect on toxicity or receptor binding (29). Similarly, a mutant in Cry1C that is lepidopteran and dipteran active, located in domain II-loop 3 (S438Y), abolished toxicity and binding to *Spodoptera littolaris* but had only a minor effect to *A. aegypti* (39). This Cry1C mutant is another example that certain domain II residues may have differential role in recognizing different receptor molecules. Our results show that residue S259 may have a differential role in binding the natural receptor or Cyt1Aa.

The data provided here indicate that membrane-inserted Cyt1Aa presents binding epitopes that are recognized by loop regions of Cry11A-domain II, which are also involved in binding to the BBMV receptor. Therefore, the interaction with Cyt1Aa could facilitate Cry11Aa toxin membrane insertion, enhancing Cry11Aa toxicity or suppressing resistance because of Cry-receptor mutations. The most common mechanisms of resistance of lepidopteran pests to Cry1A toxins are mutations affecting receptor production (40). In the case of *C. pipiens* mosquito larvae resistant to the Bin toxin, they show mutations affecting receptor assembly in the membrane (41). For Cry1A toxins, multiple receptors are involved in toxin mechanism (30). The fact that suppression of a *C. quinquefasciatus* Cry11A-resistant population with a Cyt1Aa-Cry11A mixture did not restore 100% susceptibility to Cry11A toxin (6) suggests that, in the case of mosquitoes, multiple receptors could also be involved in toxicity.

Overall, these data represent an example of an insect pathogenic bacterium that carries a toxin and also its functional receptor, promoting toxin binding to target membranes and toxicity.

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